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The International Standard for Streptokinase-Streptodornase

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The National Institute for Medical Research, London, was asked by the WHO Expert Committee on Biological Standardization to take steps to set up an international standard of streptokinase-streptodornase. This paper describes the nature and handling of the material and its establishment as the International Standard for Streptokinase-Streptodornase, with a defined potency of 3100 International Units of Streptokinase and 2400 International Units of Streptodornase per ampoule. The International Unit of Streptokinase is defined as the activity contained in 0.002090 mg of the International Standard for Streptokinase-Streptodornase. The International Unit of Streptodornase is defined as the activity contained in 0.002700 mg of the International Standard for Streptokinase-Streptodornase.

The WHO Expert Committee on Biological Standardization (1960) noted that there was a need for an international reference preparation or an international standard of streptokinase and streptodornase and asked the National Institute for Medical Research (NIMR), London, to examine preparations for their suitability to serve as a reference for purposes of assay of these two enzymes. A preparation of medium purity was selected and examined in an international collaborative assay. This paper describes the results of the collaborative study, the establishment of the material as the International Standard for Streptokinase-Streptodornase, the definition of the International Unit of Streptokinase and the International Unit of Streptodornase.

MATERIAL FOR THE PROPOSED INTERNATIONAL STANDARD

THE BULK MATERIAL

The material for the proposed international standard was obtained through the generosity of the Lederle Division of American Cyanamid Co., Pearl River, N.Y. It consisted of some 6 g of a single batch of freeze-dried powder, Lot No. 48035-154. The manufacturer supplied the following data:

Streptokinase activity, 4063 NIH units/mg (79 NIH units/μgN)
Streptodornase activity, 4080 NIH units/mg
Total N, 5.16%
Moisture content, 2.29%
Phosphate buffer Na_2HPO_4, 584 mg/g; NaH_2PO_4, 225 mg/g.

The material was received at the National Institute for Medical Research, London, in October 1961 and was stored in the dark at −10°C.

DISTRIBUTION INTO AMPOULES

In July 1962, 4.776 g of the maternal were dissolved in 4.05 litres of 0.5% (w/v) lactose in glass-distilled water. The solution was centrifuged and filtered through a membrane filter (mean pore diameter 0.45μ). The solution was then distributed in one day into some 3550 ampoules in equal (approximately 1.1 ml) amounts. The maximum difference in weight of the contents of 61 ampoules taken during the filling was ±1%. The ampoules were freeze-dried as one batch in a shelf freeze-drier and then stored over P_2O_5 in evacuated desiccators for two weeks. The desiccators were then filled three times with pure dry nitrogen and the ampoules taken out

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and sealed. The ampoules were subsequently tested for structural flaws and leaks and stored at −10°C.

The dry contents of 10 ampoules were weighed after taking precautions to prevent uptake of moisture; the average content of each ampoule was 6.48 mg and the maximum variation about this figure was ±1%. Each ampoule thus contains about 1 mg of the material and about 5.5 mg of lactose. To all intents and purposes it can be assumed that each ampoule contains the same amount as another ampoule. It cannot be assumed, however, that the streptokinase is evenly distributed within the freeze-dried plug; the powder should therefore *not* be extracted and weighed. Instead, it is intended that the entire contents of each ampoule should be dissolved in a known amount of solvent; that solution then contains the number of International Units assigned as a result of the collaborative assay.

Check assays of the streptokinase activity in the ampoules were carried out by Dr V. Houba of The Research Institute for Rheumatic Diseases, Prague, and at the National Institute for Medical Research, London. Although a small loss of activity was found, it was thought that the material was suitable for collaborative assay.

STABILITY OF THE PROPOSED INTERNATIONAL STANDARD

The stability of the proposed international standard was estimated from accelerated degradation studies of material in ampoules stored at +56°C, +37°C, +22°C, 0°C and −10°C. Comparison of the activity of samples stored for up to 12 months at the elevated temperatures with those at —10°C indicated a first-order loss of streptokinase activity; a graph of the first-order constants for activity loss plotted against the reciprocal of the absolute temperature gave a straight line, which on extrapolation to —10°C (the temperature at which the ampoules are stored) gave a maximum estimated loss in potency of under 1% per annum.

The loss of streptokinase activity in solution was determined at 10 units/ml, the concentration usually used for assay. No loss was observed in borate saline buffer solutions (pH 7.4) for four hours, or in 0.5% gelatin in buffered phosphate (pH 7.4) for 24 hours at 22°C.

THE COLLABORATIVE ASSAY

Sixteen laboratories concerned with production, control, or research on streptokinase were invited to participate in a collaborative assay of the proposed international standard; of these, twelve in five countries agreed to take part. Participants were asked to compare the streptokinase and streptodornase activities in the total contents of ampoules of the proposed international standard with those of the first British Standard (BS), the American standard issued by the National Institutes of Health (NIH) in the USA, and with any other national standard at their disposal. They were asked to perform several assays of each type of activity using any methods with which they were familiar.

Assay results were obtained from eight laboratories in five countries. These laboratories are listed in the annex; in this report each participating laboratory is referred to by a number which is not necessarily related to the order in which the names appear in the Annex.

ASSAYS OF STREPTOKINASE ACTIVITY

Methods

The assay methods used by different laboratories are listed in Table 1. The assays of fibrinolysis used by Laboratories 1, 2, 3, 4 and 5 were very similar, except for small differences in the purity of the reagents used and minor details of technique. The assay was based on the method of Christensen (1949), which depends upon a linear log/log relationship between streptokinase concentrations and the lysis times of fibrin clots formed in the presence of streptokinase. Although the absolute lysis times of the clots varied both within and between laboratories, potencies were estimated by comparison of the three-point dose-response curves obtained for both preparations using the same batch of reagents.

The method used by Laboratory 1, based on measurement of casein hydrolysis, gave results in agreement with those obtained by the fibrinolytic method. In this method the fraction of a given excess of a sample of plasminogen activated to plasmin in 10 minutes was assayed by the digestion of casein. A dose-response relationship was obtained between the plasminogen activated and the streptokinase dilution added to the activation mixture, and comparison of the potencies of proposed international standard and British Standard was made on this basis.

TABLE 1
ASSAY METHODS USED BY PARTICIPATING LABORATORIES FOR THE DETERMINATION OF STREPTOKINASE

Laboratory No.	Method	Abbreviation
1, 2, 4	Visual inspection of lysis of preformed clot of human fibrin containing plasminogen. Reagents: partially purified human fibrinogen (containing 80 % clottable protein) and human thrombin containing plasminogen. Christensen (1949)	FCLC
1, 2, 3, 5	Visual inspection of lysis of preformed clot of purified human fibrin containing purified human plasminogen. Reagents: human fibrinogen deficient in plasminogen (clottable protein 90 %), purified human plasminogen (Kline, 1953) clotted with purified bovine thrombin. Unpublished	FCLP
7	Spectrophotometric measurement of peptides soluble in urea and trichloracetic acid, liberated from preformed clot. Reagents: human plasminogen (prepared according to Kline, 1953); bovine fibrinogen free of plasminogen; bovine thrombin. Anderson (1962).	FQ
1	Spectrophotometric measurement of peptides, soluble in TCA, liberated from casein by plasmin activated by graded doses of streptokinase from excess plasminogen in specified time. Reagents: casein (Hammarsten, high nitrogen), human plasminogen (Kline, 1953). Kline (1953); Sgouris et al. (1961)	CSR
6	Determination of amount of streptokinase to give maximal activation of defined amount of plasminogen. Reagents: Casein (Hammarsten, high nitrogen), human plasminogen. Unpublished	CST

The method used by Laboratory 6 was based on the premise that the proteolytic activity developed on the complete activation of plasminogen by streptokinase under standard conditions was related to the streptokinase activity present. In this assay the amount of plasmin formed during activation by various dilutions of streptokinase was determined by the digestion of casein. It appeared that the proteolytic activity measured in this way was maximal for a certain concentration of streptokinase; greater or lesser concentrations gave less proteolytic activity. Dilutions of the preparations which showed maximum plasmin activity were considered to contain equivalent streptokinase activities.

Results

The results of the comparison oi the proposed international standard with the NIH Standard (Table 2) were, with one exception, consistent with each other. The difference between the highest and the lowest estimates was 25 % in 26 assays submitted, excluding the data of Laboratory 6. The geometric mean potency calculated from these 26 estimates, was 3109 NIH units/ampoule. No explanation could be found for the fact that the estimate obtained by Laboratory 6 was more than three times the mean potency obtained by the other participating laboratories.

When compared with the British Standard (Table 3), however, the estimated potencies of the proposed international standard were found to vary over a wide range. Variation occurred both within and between laboratories and also between methods; some degree of consistency was observed only in successive assays by one method within laboratories. The extent of the variation did not appear to bear any relationship to the method of assay or to the type of reagents used in these assays. It was thus

TABLE 2

STREPTOKINASE ACTIVITY OF THE PROPOSED INTERNATIONAL STANDARD IN TERMS OF UNITS OF THE NIH STANDARD

Method of assay [a]	Lab. No.	Estimated potency (NIH) units/ampoule	Geometric mean potency [b] (NIH units/ampoule)	
			Within laboratory	Within method
FCLC	1	3 895, 3 959, 3 805	3 886	
	2	3 050, 4 250, 3 940, 2 900 [c]	3 489	3 650
	4	3 696, 3 581	3 638	
FCLP	2	2 900	2 900	
	3	3 315, 3 048, 3 280, 3 150, 3 000, 3 425, 3 385, 3 430, 3 133, 2 981	3 209	2 813
	5	1 790, 2 370, 2 300, 2 390, 1 970	2 150	
CSR	1	3 610	3 610	3 610
CST	6	10 500	10 500	10 500

[a] Abbreviations as described in Table 1.
[b] Geometric mean potency of all 27 assays: 3253 NIH units/ampoule. Geometric mean potency excluding laboratory 6: 3109 NIH units/ampoule.
[c] Figure obtained by slightly different method.

TABLE 3

STREPTOKINASE ACTIVITY OF THE PROPOSED INTERNATIONAL STANDARD IN TERMS OF UNITS OF THE BRITISH STANDARD

Method of Assay [a]	Lab. No.	Estimated potency (British units/ampoule)	Geometric mean potency (British units/ampoule)	
			Within laboratory	Within method
FCLC	1	940, 990, 1 065, 979, 943, 960	979	
	2	2 150, 2 550, 2 990, 1 730 [b]	2 308	1 434
	4	1 643, 1 802, 1 463	1 630	
FCLP	1	1 012, 975, 950, 1 034, 983, 959	985	
	2	5 240, 4 950	5 093	2 023
	3	3 230, 2 984, 3 189, 2 633, 2 500, 2 707, 3 144	2 899	
FQ	7	824, 846, 806	825	825
CSR	1	960	960	960
CST	6	840	840	840

[a] Abbreviations as described in Table 1.
[b] Figure obtained by slightly different method.

not possible to attempt to define the potency of the streptokinase activity of the proposed international standard in terms of the British Standard. The latter is of material of low specific activity and it seems likely that the failure to obtain consistent valid results is simply a reflection of the difference in purity of the two preparations.

ASSAYS OF STREPTODORNASE ACTIVITY

Methods

The methods used by different laboratories are listed in Table 4. Laboratories 1 and 2 followed the depolymerization of desoxyribonucleic acid (DNA) by streptodornase by measurement of the increase in optical density at 260 mμ in the spectrophotometric assay according to the method of Kunitz (1950). However, Laboratory 2 based comparisons of enzyme activity on rates of digestion of DNA at one enzyme dilution only, whereas Laboratory 1 performed assays at two dilutions of the proposed international standard and the British Standard. The potencies in both assays were obtained by comparing the slopes of the linear part of the curves obtained by plotting the increase in optical density at 260 mμ against time, in a mixture of enzyme and highly polymerized DNA in the presence of optimal concentrations of Ca^{++} and Mg^{++} ions at pH 7.5 (tris-(hydroxymethyl) amino methane HCl buffer).

The viscometric methods used by Laboratories 3 and 4 for the comparison of activity differed only in the method of measuring viscosity; the former laboratory used a semi-auomatic capillary viscometer, and the latter used Ostwald viscometers.

The desoxyribonuclease activities were compared on the basis of a linear relationship between the enzyme concentration and the reciprocal relative viscosity of the enzyme/substrate mixture after 50 minutes' digestion. The curve was constructed from measurements of relative viscosities taken every five minutes up to 50 minutes. A value (K_{50}) for each enzyme preparation was derived by linear extrapolation of the initial (linear) part of the curve plotting this relationship.

TABLE 4

STREPTODORNASE ACTIVITY OF THE PROPOSED INTERNATIONAL STANDARD IN TERMS OF UNITS OF THE BRITISH STANDARD

Method of assay	Lab. No.	Estimated potency (British units/ampoule)	Geometric mean potency [a] (British units/ampoule)	
			Within laboratory	Within method
Increase in optical density at 260 mμ (Kunitz, 1950)	1	2 400, 2 600, 2 080	2 350	
	2	2 135, 2 206, 2 395, 2 130, 2 057, 2 064, 2 348, 2 270, 2 347	2 214	2247
Viscometric, based on method of Christensen (1949)	3	2 340, 2 326	2 333	2 805
	4	3 403, 3 049, 3 077	3 173	
Uncoupling of methyl-green complex (Kurnick, 1953)	8	2 450	2 450	2 450

[a] Geometric mean potency of all 18 assays: 2401 British units/ampoule. Geometric mean potency excluding Laboratory 4: 2271 British units/ampoule.

Laboratory 4 reported data for K_{50} at several dilutions of the proposed international standard and the British Standard and confirmed a linear relationship between K_{50} and enzyme concentration for the dilutions of the two standards. Laboratory 3 supplied data of determinations at one enzyme concentration only.

The relative potencies of the proposed international standard and the British Standard were determined at different dilutions using the methyl-green method (Kurnick, 1953) by Laboratory 8. This laboratory noted a non-specific uncoupling of methyl green from the DNA/Mg/methyl-green complex by low dilutions of the proposed international standard and British Standard. The effect could be reduced by further dilutions, although the British Standard continued to show a much greater tendency to uncouple the dye at dilutions at which the proposed international standard behaved satisfactorily. The relative potencies were measured by comparison of the decrease in optical density at 640 mμ of samples of DNA/Mg/methyl-green after digestion for a standard time and after storage overnight in the dark. Only results obtained using high dilutions of the proposed international standard and the British Standard are presented in Table 4.

Results

The only material with a defined streptodornase activity which was studied by all participants was the British Standard. The results of assays of the proposed international standard in terms of it are given in Table 4.

The results within each laboratory were homogeneous, and it can be seen that those obtained by Laboratories 1, 2, 3 and 8 agreed closely with one another, although three different methods were used for following the depolymerization of DNA.

The over-all geometric mean potency of the proposed international standard in terms of units of the British Standard based on all estimations was 2401 units/ampoule. The mean potency obtained by Laboratory 4 (3173) was substantially above the mean potency of all the other results (2271).

Laboratory 4 prepared its own substrate DNA (according to the method described by Zamenhof (1958), whereas Laboratory 3 obtained a commercial supply of DNA; otherwise Laboratories 3 and 4 used the same method of assay.

DISCUSSION

An activity other than its ability to activate plasminogen of certain mammalian species has not yet been demonstrated for streptokinase. The mechanism of this activation is complex and not yet clear but it is believed to involve several stages, at least some of which appear to behave like simple enzyme systems. The initial interaction between plasminogen and streptokinase is rapid and in this respect is not characteristic of an enzyme-mediated reaction.

Furthermore, inhibitors of streptokinase and of intermediate activities involved in plasminogen activation are known to exist and may contaminate some of the " purified " preparations of plasminogen used in streptokinase assays.

The complexity of these interactions makes it impossible to assay streptokinase with methods based on measurements of any particular absolute effect, such as the time of lysis of a plasminogen-rich fibrin clot, or the rate of proteolysis of casein, and thus renders it necessary to measure potency by comparative bioassay.

All the results submitted, except those from one laboratory, were obtained by methods which provided a graded dose-response relationship. Not all, however, were amenable to statistical tests; since laboratories did not carry out multiple replicates on the same solution from the same ampoule, analysis of variance within an assay was not possible and the individual estimates of potency are reported.

Many of the assays that were repeated were carried out using slightly different techniques or substrates and the conditions were thus not sufficiently homogeneous for it to be suitable to combine the estimates. For this reason weighted mean potencies have not been calculated and the geometric mean potencies only are reported. The comparison of activities involved no initial assumption of the mode of interaction of streptokinase and plasminogen. The results from Laboratory 6, however, were different in so far as they were based on matching a concentration which produced a certain effect (maximum activation of plasminogen), and thus did not give a graded dose-response relationship. The estimates of potency obtained by this method gave a figure which was more than three times the geometric mean potency of all other determinations in terms of the NIH Standard. For these reasons the figure has not been included in the calculation of the over-all mean potency.

The proposed international standard and the British Standard were compared in further studies using a plate diffusion method (P. L. Walton—to be published) developed from the fibrin plate assay of Astrup & Mullertz (1952). The method can be carried out with an assay design to give data susceptible to formal statistical analysis. The slopes of the dose-response were significantly different for these two preparations; since the precision of the method is good it is possible that it may reveal further slope differences between different preparations of streptokinase.

The estimates of the streptodornase activity of the proposed international standard in terms of the British Standard agreed closely with one another (Table 4) when three different methods were used for the determination of the rate of depolymerization of DNA.

ESTABLISHMENT OF THE INTERNATIONAL STANDARD AND DEFINITION OF INTERNATIONAL UNITS

The ratio of streptokinase activity to streptodornase activity is not expected to be the same in any two preparations. Moreover, there is no evidence that different concentrations of one enzyme will affect the assay of the other. There is thus no particular need to relate the unitage of the one activity to the other. The assignment of a different unitage for each enzyme activity may indeed serve to emphasize that they are not interdependent.

Since the only national standard with which a number of satisfactory and homogeneous potency estimates for streptokinase were obtained was the American (NIH) one, the unitage of the proposed international standard was related to the American unit. The geometric mean of the estimates was 3109 NIH units/ampoule. It was agreed by all participants that this figure should be rounded off to 3100 units/ampoule, since it is intended that in use the entire contents of an ampoule be dissolved in a known volume. The mean weight of dry contents was 6.48 mg/ampoule. In accordance with authority given by the WHO Expert Committee on Biological Standardization (1964), the material has been established as the International Standard for Streptokinase-Streptodornase. With the agreement of the participants, the International Unit of Streptokinase was defined as the activity contained in 0.002090 mg of the International Standard for Streptokinase-Streptodornase.

The streptodornase activity of the International Standard was assayed only in terms of the British Standard and the International Unit was therefore made similar to the British unit; the estimates of potency were homogeneous and the geometric mean

potency was 2401 units/ampoule, which was rounded off to 2400 units/ampoule. The International Unit of Streptodornase was defined as the activity contained in 0.002700 mg of the International Standard for Streptokinase-Streptodornase.

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RÉSUMÉ

Dans son treizième rapport (1960), le Comité OMS d'experts de la Standardisation biologique a estimé que l'établissement d'une préparation internationale de référence ou d'un étalon international de streptokinase et de streptodornase répondait à un besoin. Il a donc invité le National Institute for Medical Research de Londres à examiner un certain nombre de préparations susceptibles d'être utilisées comme étalon commun pour le titrage de ces deux enzymes.

Un lot de 6 g d'une préparation de pureté moyenne, reçu en 1961, a été sélectionné, réparti en ampoules, et examiné au cours d'une étude collective menée dans 8 laboratoires de cinq pays. Plusieurs méthodes ont été utilisées pour comparer l'activité de cette préparation à celle d'étalons nationaux. A la suite de ces essais, la préparation proposée a été constituée en étalon international de streptokinase-streptodornase. Avec l'accord des laboratoires participants, sa valeur a été fixée à 3100 unités internationales de streptokinase et à 2400 unités internationales de streptodornase par ampoule. L'unité internationale de streptokinase a été définie comme l'activité de 0,002090 mg de l'étalon international de streptokinase-streptodornase. L'unité internationale de streptodornase a été définie comme l'activité de 0,002700 mg de l'étalon international de streptokinase-streptodornase.

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Annex

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